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Original Article

Establishment and Validation of LC-MS/MS Technique for Pafolacianine Quantification in Rat Plasma, with Application to Pharmacokinetic Assessment

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ABSTRACT

Background: Establishing and validating a sensitive and accurate LC-MS method for quantifying pafolacianine in rat plasma was the primary objective of this study. Phenylalanine was used as the internal standard, and the validation procedure adhered to the protocols specified by the Food and Drug Administration of the United States. **Materials and Methods:** This article presents an overview of the bioanalytical LC-MS method, utilizing an Inertsil ODS column (150 mm x 4.6 mm, 3.5 µm) and an organic mobile phase comprising acetonitrile and 0.1% formic acid buffer in a ratio of 40:60. **Results:** The calibration curve for pafolacianine exhibited a linearity range of 5-100 ng/mL (r2=0.9999). Liquid-liquid extraction was employed to recover pafolacianine from rat plasma, resulting in recovery percentages of 100%, 99.7%, and 99.8% at three different concentration levels. Pafolacianine remained stable during storage under various conditions (three freeze-thaw cycles, benchtop, autosampler, short-term, and long-term storage). Pharmacokinetic analysis yielded key parameters, including a half-life of 9.6 m and a time to reach a maximum concentration of 5 m. Pafolacianine and phenylalanine were identified using proton adducts in the LC-MS analysis at *m/z* 1326.3/574.6 and 166.08/144.8, respectively, by employing positive mode multiple reaction monitoring. **Conclusion:** This comprehensive evaluation demonstrates that the method meets stringent criteria for system specificity, linearity, and accuracy, all well within the predefined acceptance limits. Its adaptability for the precise determination of pafolacianine positions it as an invaluable tool in the field of bioanalysis, expanding its clinical utility.

Keywords: Pafolacianine, Phenylalanine, Pharmacokinetics, Bio-analytical, Rat plasma, LC-MS/MS.

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INTRODUCTION

Ovarian cancer ranks as the second most prevalent gynecologic cancer, causing more female fatalities than any other form of cancer affecting the female reproductive system. Unfortunately, early detection remains a challenge, with only 20% of ovarian cancer cases identified in their initial stages, often due to the absence of noticeable symptoms or their resemblance to common bladder, bowel, or gastrointestinal conditions.[1] Surgical resection is often a crucial part of cancer treatment, and advancements in technologies aiding surgeons to identify and remove cancerous lesions can significantly impact patient outcomes. Pafolacianine (PC) is one such optical imaging drug designed to assist surgeons by serving as an additional aid in identifying and illuminating ovarian cancer lesions, including those situated in typically hard-to-spot regions, when employed during surgeries.[2] PC comprises an amino acid connector connecting a Near-Infrared (NIR) cyanine dye with a folate analogue.[3] This unique amalgamation empowers PC to specifically attach to cancer cells that exhibit an elevated expression of FRα. Upon exposure to suitable near-infrared light, PC emits fluorescence, enabling the visualisation and pinpointing of cancerous tissues following standard intravenous administration.[4] This can occur as swiftly as one hour before surgery, significantly assisting surgeons in achieving more precise tumour removal. PC is chemically composed of 2-{(E)-2-[(3E)-2-(4-{2-[(4-{[(2-Amino-4-oxo-3,4-dihydro-6pteridinyl)methyl]aminobenzoyl)amino]-2-carboxyethyl}}phenoxy)-3-{(2E)-2-[3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)-1,3-dihydro-2H-indol-2-ylidene]ethylidene}-1-cyclohexen-1-yl]vinyl}-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium-5-sulfonate. PC is made up of molecules with the formula

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$C_{61}H6_7N_9O_{17}S_4$ and a weight of 1326. Figure 1 is a picture of its structural form. A crucial aspect of evaluating the safety of any drug understands its bioavailability.[5] Patients and healthcare professionals should recognise the importance of bioavailability and stay well-informed about cancer care, seeking guidance from experts to determine the most suitable treatment for individual cases**.** On November 29, 2021, the FDA granted approval for PC, which is now available under the brand name Cytalux.[6] However, the amount of information that is now available concerning the analytical approaches that are utilised to identify PC in biological materials and carry out pharmacokinetic evaluations in animal models, is noticeably limited. Nevertheless, the robust and highly precise analytical technique of LC-MS/MS can be harnessed for the accurate quantification of drugs in biological specimens, including plasma.[7,8] Hence, research has been dedicated to investigating the pharmacokinetics of PC as well as developing, validating, and implementing a comprehensive bioanalytical approach.[9,10] PC falls within the category of organic compounds referred to as phenylalanine derivatives, making Phenylalanine (PA) the most suitable choice as an internal reference standard. Employing LC-MS/MS for studying pharmacokinetic data significantly enhances sensitivity and productivity levels. We believe that this is the very first time that a bioanalytical approach has been developed specifically for analysing PC.

MATERIALS AND METHODS

Method development

Chemicals and Reagents

A PC sample with a purity of 99.80% was obtained from Biocon Ltd., in Bengaluru, India. Chemicals and reagents, including LCMS-grade acetonitrile, LCMS-grade methanol, and Water purified by the Milli-Q water purification system to meet the standards for HPLC-grade quality, were exclusively sourced from Merck Chemical Division in Mumbai, India. The utilisation of HPLC-grade water maintained the utmost level of water purity consistently throughout this study. The plasma samples utilised in this research were procured from Bioneeds in Bangalore, India.

LC-MS/MS instrument and conditions

The Waters Alliance E-2695, equipped with a high-speed sample handler, column oven, and de-aerator, was used for the experimental procedures. Mass spectrometric analysis was carried out using a SCIEX QTRAP 5500 mass spectrometer, and data processing was conducted using SCIEX software. Detection was achieved through electrospray ionisation in positive mode with unit resolution. The quantification of PC was performed using MRM mode. At *m/z* 1326.3, the precursor ion MH^+ was observed for PC, while a specific fragment ion was detected at *m/z* 574.6. Concerning PA, the precursor ion MH^+ was observed at a *m/z* of 166.08, and the fragment ion at 144.8 as illustrated in Figure 2. The mass spectrometry settings were fine-tuned by setting the source temperature to 550°C, the heater gas to 40 psi, the nebulizer gas to 50 psi, the shielding gas to 30 psi, the collision gas for fragmentation to 2 psi, the electrospray voltage to 5500 V, and the nebulizer gas flow rate to 600 mL/ m without splitting. The other settings, entry voltage at 10 V, delustering potential at 40 V, and collision energy at 14 V were applied identically in both cases of PC and PA.

Chromatographic Conditions

For the chromatographic analysis, an Inertsil ODS column measuring 150 mm x 4.6 mm with a particle size of 3.5 µm was selected. The mobile phase was a combination of acetonitrile and a buffer containing 0.1% formic acid, and it was given at a flow rate of 1 mL/m. The ratio of the two components was 40:60. The mobile phase serves the dual purpose of being the diluent itself. Throughout the entirety of the analysis, the temperature of the column did not deviate from the standard setting. PA was chosen as the internal standard due to its suitability in terms of

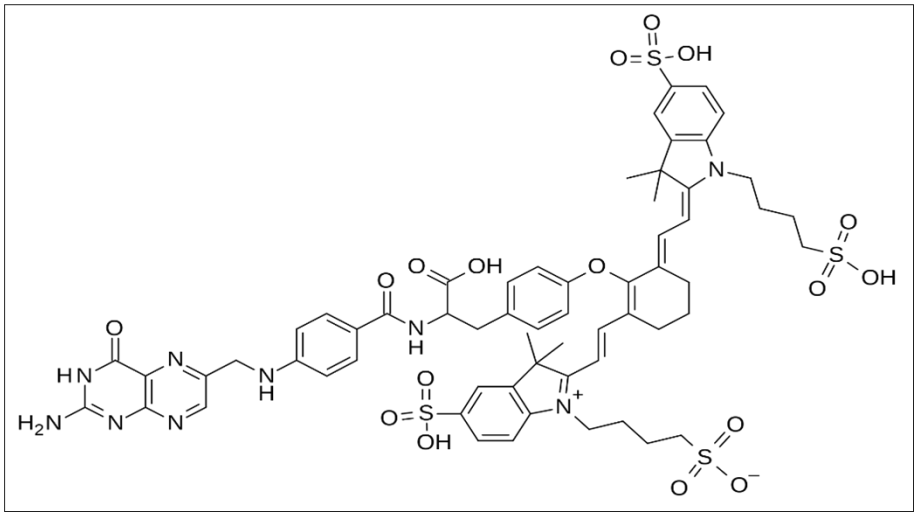

Figure 1: Chemical structure of PC.

chromatographic performance and extractability. Each sample was injected into a volume of 10 µL. During chromatographic analysis, the retention time for PA was approximately 2.32 m, while for PC, it was approximately 3.084 min. Consequently, each analysis had an overall runtime of approximately 5 m.

Development of Calibration Standards and Quality Control Samples

Standard stock solutions of PC and PA were formulated at a concentration of 200 ng/mL. A working standard solution of 50 ng/mL was produced by suitably diluting the PC master stock solution, originally at 200 ng/mL, using the mobile phase. A working IS solution of 50 ng/mL was prepared in the mobile

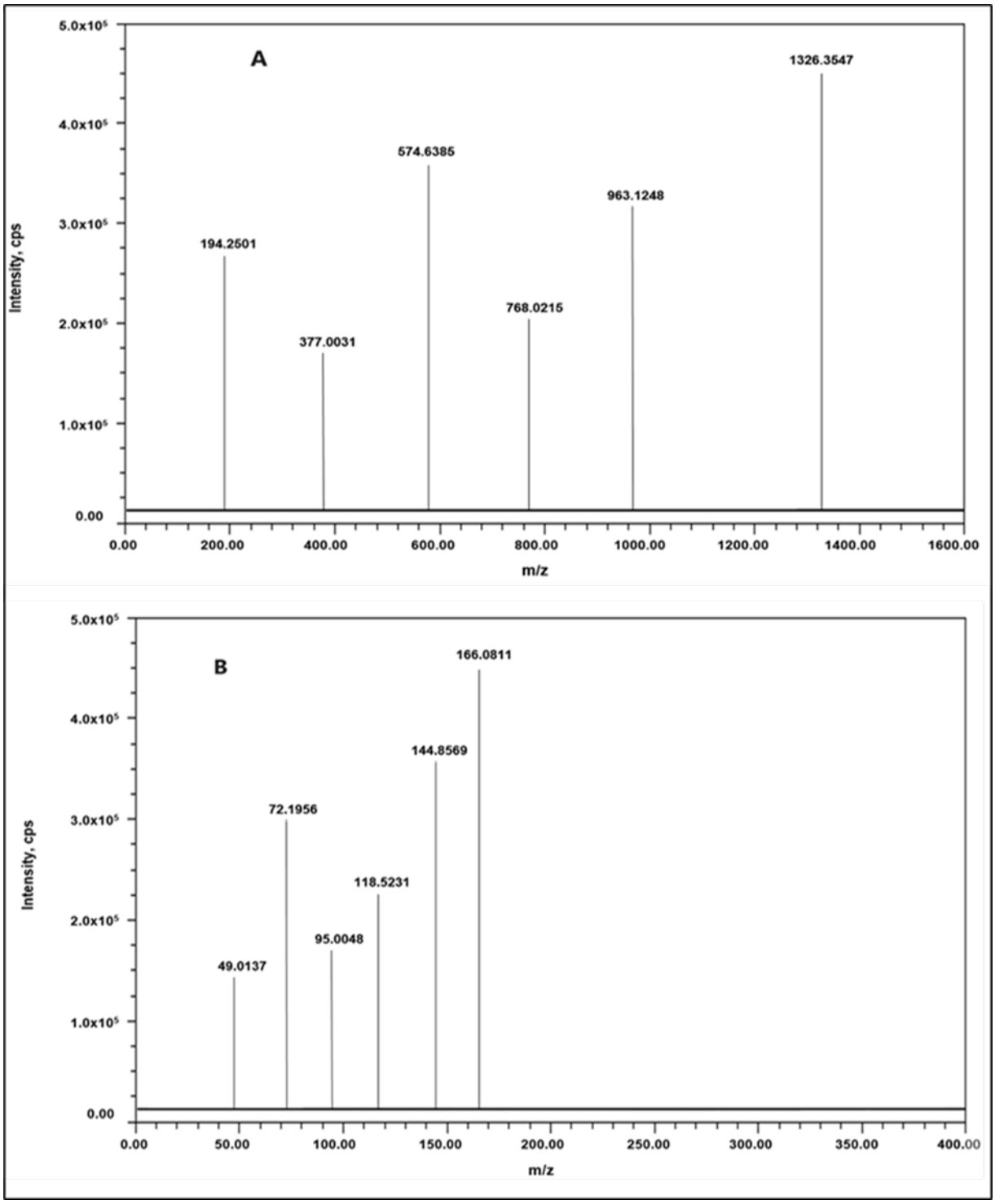

Figure 2: Multiple reactions monitoring of (A) PC (B) PA in positive polarity: Analogous precursor ion and daughter ion with the highest intensity (*m/z*).

phase. The PC standard solutions that are suitably diluted from the master stock solution were then added to drug-free rat plasma. This result in PC concentrations of 5, 12.5, 25.00, 37.5, 50, 62.5, 75, and 100 ng/mL for the analytical calibration standards. In addition to calibration standards, QC standards were established with specific PC concentrations of 5, 25, 50, and 75 ng/mL. The purpose of these quality control samples is to track the development and dependability of the analytical method. Every calibrated and quality control standard that was prepared was preserved in a freezer set to -30°C.

Sample preparation for PC and PA from Rat Plasma

Liquid-liquid extraction was employed for the isolation of PC and its respective internal standard PA from rat plasma samples. To begin, a volume of 500 µL of IS (stock solution) was added to 200 µL of plasma (corresponding concentrations), ensuring that the mixture was thoroughly combined. Following this, 300 µL of acetonitrile was introduced, and the mixture was vigorously vortexed. Subsequently, the mixture was centrifuged at 4000 RPM for duration of 15 to 20 m, resulting in the separation of the supernatant solution. The liquid above was cautiously moved to a new tube with 2 mL of 2 M $MgSO_4$ and then subjected to vortexing. For the preparation of the samples for analysis, the supernatant from each sample was meticulously transferred into appropriately labeled RIA vials. Subsequently, the solvent underwent evaporation at 40°C until complete desiccation was achieved. Following this, the desiccated samples were reconstituted with 1000 µL of the diluent (mobile phase), undergoing a brief, vigorous mixing process for homogeneity. Finally, these reconstituted samples were transferred into autosampler vials, rendering them ready for injection into the chromatograph.

Method Validation

Linearity

This study exhibits the linearity of detector response by utilising eight different concentrations of a PC solution, ranging from 5-100 ng/mL. The range of concentrations was chosen to demonstrate the linearity of the detector response. The experiment utilised a method of extraction that was consistent with the goal of validating the linearity of the detector's response while assessing varied concentrations of PC solutions that were put into rat plasma. As the Internal Standard, we utilised a solution with a constant concentration of 50 ng/mL. The HPLC system was then fed with the sample concentrations that had been determined as a consequence. The data that was collected, which represented the ratio of PC peak area to PA peak area, was used to generate a correlation plot relating the ratio with PC concentration in ng/mL. The correlation plot was linked to the ratio. The regression analysis was used to determine important parameters such as the correlation coefficient, slope, intercept, LOD, and LOQ

Selectivity and Specificity

By corresponding their distinct retention times to the corresponding MRM responses, the chromatographic peaks of PC and PA were discerned. A criterion was established to evaluate selectivity: the peak area of PC at its specified retention time in blank samples must not surpass 20% of the mean peak area of the PC's Limit of LOQ. The mean peak area of the PA's LOQ should not exceed 5% of the peak area at its retention time in blank samples.

Precision and accuracy

Intraday and interday precision were determined through a rigorous evaluation of six replicates of each concentration at the), LLOQ (5 ng/mL), LQC (25 ng/mL) MQC (50 ng/mL), and HQC (75 ng/mL). The evaluation of inter-day precision spanned multiple days, whereas intra-day precision was determined by analysing these replicates on the same day. In order to determine the coefficient of variation (RSD), comparisons were made between the measured and expected true responses at the QC levels using the resulting area response ratio values. A criterion of 20% accuracy was established for the LLOQ level, signifying that the measured values ought not to exhibit a greater than 20% deviation from the true values. A 15% acceptable accuracy criterion was subsequently established for the HQC, MQC, and LQC levels.

% Recovery

In the assessment of PC and PA extraction recovery from rat plasma, a rigorous procedure was conducted, involving the analysis of six replicate injections of QC samples. These QC samples included LQC, MQC, and HQC with corresponding concentrations of 25, 50, and 75 ng/mL. This determination was made by comparing the peak areas derived from the extracted plasma samples with the peak areas from a standard solution that had been spiked with the blank plasma residue. It is imperative to note that a recovery rate exceeding 50% was the established criterion for adequacy in achieving the requisite sensitivity in this analytical process.

Matrix effect

In order to evaluate the impact of the matrix, six batches of empty biological matrices were created. Each batch was then mixed with the pure standard at two different concentration levels, specifically the LQC and HQC levels, with each concentration level being tested three times. Subsequently, the spiked samples were compared to the neat standards of identical concentration by means of alternate injections. In order to determine the dependability of the matrix factor, total accuracy was used as a primary measure. The level of precision was quantified as CV%. The acceptable threshold for CV% was established at 15% or below, which is worth mentioning.

Stability

The experimental design comprised six repetitions for each of the three concentration levels (LQC, MQC, and HQC). In accordance with FDA regulations, the stability of an analyte is assessed by monitoring the extent of variation in its concentration within a 15% threshold. To evaluate resistance to repeat freezing and thawing, freeze-thaw stability was determined by subjecting samples to three consecutive defrost cycles at -31°C, followed by a comparison with freshly injected internal control samples after the third cycle. The bench-top stability study involved storing QC samples at these concentrations at room temperature for 24 hr. Subsequently, these samples were compared to plasma extracts that were immediately analysed. To ensure short-term stability, six replicates of LQC, MQC, and HQC samples were maintained at 7°C for seven days. After examining the long-term stability for 1, 7, 14, 21, and 28 days, samples were analysed. To evaluate the analyte's stability under prolonged exposure, the auto-sampler stability test utilised three samples of the specified QC levels that were stored in an auto-sampler at 15°C for durations spanning from 0 to 24 hr. In the dry and moist extract plasma sample evaluation, the stability of samples stored at ambient temperature for specified times 12 hr and 18 hr within the range of 2-8°C was compared. The evaluation encompassed a comparison with newly extracted samples to determine the stability of the analyte by ensuring that the RSD remained below 15%.

Application to the pharmacokinetic study of PC in rat plasma

The validated analytical approach delineated in this investigation has effectively been utilized for the quantitative evaluation of PC concentrations in rat plasma. The procedural aspects pertaining to animal experimentation were formally presented to the Committee for Control and Supervision of Experimentation on Animals (CPCSEA) and subsequently obtained approval from the duly constituted Institutional Animal Ethics Committee (Registration Number 1250/ PO/ RcBi/ S/ 21 /CPCSEA). Male Sprague-Dawley rats sourced from Bioneeds, Bangalore were utilized as test subjects, with PC administered intravenously at a concentration of 0.033 mg/kg via the left femoral vein. Blood samples were systematically collected at specified intervals ranging from 5 to 60 m post-administration, inclusive of a predose sample, to evaluate potential plasma interferences. These samples were meticulously gathered using K_2EDTA- within a controlled temperature range of 2-8°C. Plasma was isolated from these samples by centrifugation at 3000 rpm. Subsequently, the plasma samples were preserved at -30°C until analysis, following which pharmacokinetic parameters were computed leveraging WinNonlins software version 5.2. The analysis involved the supplementation of plasma samples with the internal standard PA, which were concurrently processed alongside QC samples. These stringent methodologies have provided significant insights into the pharmacokinetic profile of PC.

RESULTS

Method validation

Linearity

The evaluation of linearity was conducted by creating calibration curves plotting the ARR (PC/PA) against PC concentration. The calibration demonstrated linear behavior over a concentration range from 5 to 100 ng/mL. Statistical analysis of the data resulted in a high correlation coefficient (r^2) value of 0.9999. Additionally, the determined intercept and slope were found to be 0.02541 and 0.0240, respectively. The LOD and LOQ were calculated as 2 ng/mL and 6 ng/mL, respectively. Refer to Figure 3 for the visualisation of the calibration plot.

Selectivity and Specificity

The utilisation of the MRM function to analyse PC and PA yielded extremely selective results, which was confirmed by the absence of interference in the chromatograms of rat plasma at retention times of PC and PA. The chromatograms of the blank and blank spiked with LLOQ, LQC, MQC, and HQC samples along with ISD are displayed in Figure 4.

Accuracy and precision

In the evaluation of intraday accuracy, the accuracy percentages for the specified concentrations across the four concentration checkpoints of LLQC, LQC, MQC, and HQC were determined to be 90.0, 98.77, 100.14, and 100.96. Similarly, in the inter-day precision study, the accuracy percentages for these concentrations were consistently observed to be 93.05, 99.44, 99.14, and 100.96, as shown in Table 1.

Recovery

Figuring out PC recovery involved directly comparing the variance in peak area ratios between plasma and solvent samples. This assessment was conducted at three distinct concentrations: 25 ng/mL, 50 ng/mL, and 75 ng/mL, yielding recovery percentages of 100, 99.7, and 99.8, respectively. It is noteworthy that the RSD remained well within the acceptable limits.

Matrix Interference

The mean percent accuracy for the matrix effect was calculated to be 99.9% and 98.4%, respectively, which falls within the permissible range of 80-120%. The percent SD of the substance at both the LQC and HQC levels was acceptable. As summarised in Table 2, this indicates that the effect of the matrix on the ionisation of the analyte is within the acceptable range.

Stability

The precision of PC sample accuracy was rigorously ascertained via an exhaustive bench-top stability investigation; the resulting values of 98.5, 99.4, and 97.3 correspond to distinct

levels. Additionally, at the LQC, MQC, and HQC levels, the accuracy pertaining to freeze-thaw stability was assessed; the corresponding accuracy values were 100.8, 99.2, and 100.1. The results obtained from assessing the samples' stability in the short and long term, as indicated by the RSD remaining within 15% of the predetermined acceptability threshold, demonstrate that these samples maintained their stability for a maximum of 28 days. The consistency of auto-sampler outcomes, which include values of 98.3, 98.5, and 98.7, highlights the superior stability exhibited by processed samples after undergoing auto-sampling in comparison to freshly prepared samples. The information presented in Table 3 illustrates the overall stability test results, which indicate that the PC samples remain within the permissible range of variation throughout the entire analysis procedure.

Pharmacokinetic investigations

A formal investigation of pharmacokinetic parameters was conducted following the administration of a sole PC injection dose in six rodents, employing a non-compartmental timing analysis approach. Plasma concentrations of PC were meticulously measured at specified intervals of 5 m, 10 m, 20 m, 30 m, 40 m, 50 m, and 60 m post-dosage, and the mean of these collective concentration-time profiles are visually depicted in Figure 5. The outcomes of this investigation yielded crucial pharmacokinetic parameters: C_{max} of 45.8±0.28, T_{max} at 0.08±0.002 h, $T_{1/2}$ of 0.16±0.01 h, AUC_{0-t} at 780±5.4 ng h mL^{-1}, $AUC_{0-\infty}$ at 780.3±5.4 ng h mL^{-1}, Ke of 4.43±0.07 h^{-1}, and C_0 of 78.3±1.24. The study also revealed a V_d of 421.5±6.6 l kg^{-1} and a Cl of 1.87±0.01 l h^{-1}. A comprehensive summary in Table 4 underscores the method's effectiveness for bioanalytical investigations while providing valuable data for preclinical and pharmacokinetic research endeavors.

DISCUSSION

In the quest for efficient analysis, the optimisation of LC-MS conditions assumes importance, which involves a series of deliberate trials to fine-tune chromatographic parameters, particularly those pertaining to the mobile and stationary phases. The initial endeavors, employing a Symmetry C_{18} column (150x4.6 mm, 3.5 µ), when paired with a mobile solvent comprising an 80:20 ratio of acetonitrile to formic acid buffer, resulted in plate count values deviating from the intended limits. Subsequently, when the mobile phase composition was altered to a 70:30 ratio, the

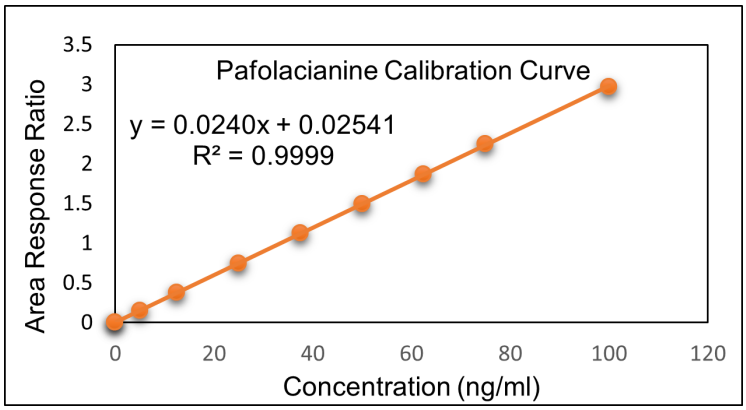

Figure 3: Calibration curve for PC.

Table 1: Precision and accuracy evaluation of PC.

Intraday Precision	SAMPLE-QC No						Mean ARR	Mean Area±SD	RSD	Accuracy
	1	2	3	4	5	6				
LLQC	4.37	4.23	4.39	4.30	4.33	3.92	0.12	4.26±0.17	0.04	90.00
LQC	24.6	24.7	24.7	24.5	24.9	24.6	0.60	24.7±0.17	0.01	98.77
MQC	49.3	49.5	49.6	49.3	49.4	49.3	1.19	1.21±0.04	0.03	100.14
HQC	75.1	74.9	74.9	75.0	75.2	75.2	1.79	1.81±0.03	0.04	100.96
Interday Precision	1	2	3	4	5	6	Mean ARR	Mean Area± SD	RSD	Accuracy
LLQC	5.09	4.73	4.53	4.12	4.80	4.64	0.13	4.65±0.3	0.07	93.05
LQC	24.9	24.5	25.1	24.6	25.5	24.6	0.60	24.9±0.39	0.02	99.44
MQC	48.8	49.3	50.3	49.1	49.2	50.7	1.20	49.6±0.77	0.02	99.14
HQC	75.3	74.7	79.0	72.9	49.4	79.3	1.83	76.4±2.53	0.03	100.96

*Mean n=The average of 6 determinations; ARR represents the ratio of area responses.

Table 2: Matrix Effects of PC.

	HQC	LQC
Plasma Lots	**Mean Concentration±SD**	**Mean concentration±SD**
LOT-1	74.92±0.14	24.56±0.092
LOT-2	75.21±0.06	24.53±0.133
LOT-3	74.97±0.35	24.51±0.122
LOT-4	74.70±0.20	24.77±0.082
LOT-5	75.17±0.33	24.66±0.164
LOT-6	74.97±0.57	24.61±0.158
% Accuracy	99.9	98.4

Mean n=3 determinations.

first chromatographic peak exhibited an unfortunate tendency to split, further complicating the quest for optimal separation. Not giving up, the experiments went on, this time using an Inertsil ODS column (150x4.6 mm, 3.5 µ) and a mobile phase of 70:30 acetonitrile to formic acid buffer. This arrangement introduced an unknown peak that emerged. Meanwhile, shifts in the mobile phase composition, specifically acetonitrile and formic acid buffer at ratios of 60:40 and 50:50, revealed inadequacies in baseline quality and inter-peak resolution, respectively. The turning point in this optimisation journey arrived with the adoption of an eluent mixture at a 40:60 ratio of acetonitrile and a 0.1% formic acid buffer. Under these conditions, utilising the Inertsil ODS column, an exquisite balance between separation and elution was achieved. With a judicious rate of flow of 1 mL/ m and a sample volume of 10 µL, the chromatographic system delivered the

Table 3: PC Stability findings in rat plasma.

Stability conditions	Quality Control Points	Average measured Concentration (ng/mL)±SD	% Recovery	RSD
Room temperature stability within 4-24 h on the bench.	LQC (25 ng/mL)	24.6±0.2	98.5	0.01
	MQC (50 ng/mL)	49.2±0.1	99.4	0.02
	HQC (75 ng/mL)	74.9±0.1	97.3	0.05
Freeze-thaw stability (Frozen at -31°C thawed thrice).	LQC (25 ng/mL)	25.2±0.5	100.8	0.02
	MQC (50 ng/mL)	49.6±0.8	99.2	0.02
	HQC (75 ng/mL)	75.1±0.8	100.1	0.01
Short-term stability (7 days at 7°C).	LQC (25 ng/mL)	24.5±0.6	98.0	0.02
	MQC (50 ng/mL)	48.2±0.9	96.3	0.02
	HQC (75 ng/mL)	75.3±1.6	100.3	0.02
Long Term Stability (After 28 days).	LQC (25 ng/mL)	24.6±0.2	90.1	0.07
	MQC (50 ng/mL)	45.2±2.2	90.4	0.05
	HQC (75 ng/mL)	67.5±1.7	90.0	0.03
Autosampler stability at 15°C from 0 to 24 hr.	LQC (25 ng/mL)	24.6±0.2	98.3	0.01
	MQC (50 ng/mL)	49.3±0.3	98.5	0.01
	HQC (75 ng/mL)	74.8±0.4	98.7	0.01
Wet extract stability (12 hr).	LQC (25 ng/mL)	24.6±0.9	98.3	0.04
	MQC (50 ng/mL)	49.5±0.5	98.9	0.01
	HQC (75 ng/mL)	74.4±0.8	99.2	0.01
(18 hr)	LQC (25 ng/mL)	24.5±0.2	97.9	0.01
	MQC (50 ng/mL)	48.8±0.6	97.6	0.01
	HQC (75 ng/mL)	74.1±0.9	98.8	0.01
Dry extract stability (12 hr).	LQC (25 ng/mL)	24.6±0.1	98.4	0.01
	MQC (50 ng/mL)	49.2±0.1	98.4	0.02
	HQC (75 ng/mL)	74.7±0.1	98.6	0.01
(18 hr)	LQC (25 ng/mL)	24.6±0.3	98.4	0.01
	MQC (50 ng/mL)	49.2±0.4	98.4	0.01
	HQC (75 ng/mL)	74.3±1.0	99.0	0.01

Mean n is the average from 6 measurements.

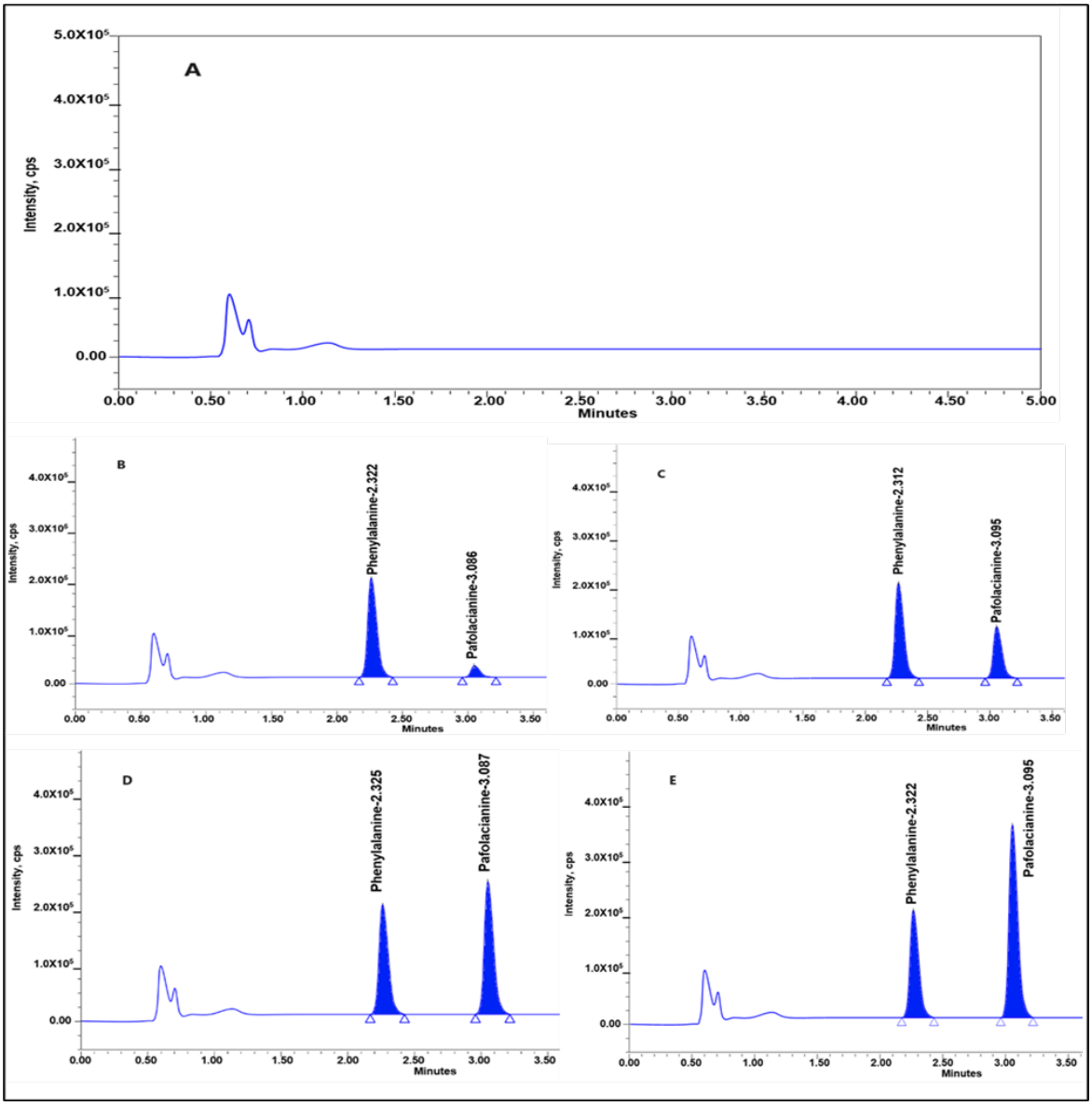

Figure 4: A). Blank plasma chromatogram devoid of any interferences; Representative chromatograms for rat plasma samples spiked with PC B) LLOQ: 5 ng/mL, (C) LQC: 25 ng/mL, (D) MQC: 50 ng/mL, and (E) HQC: 75 ng/mL.

Table 4: Mean pharmacokinetic parameter values of PC following intravenous administration in rats with standard deviation.

Kinetic Parameters	Mean*±SD	Units
C_{max}	45.8±0.28	ng/mL
T_{max}	0.08±0.002	h
K_e	4.43±0.07	h^{-1}
$T_{1/2}$	0.16±0.01	h
AUC_{0-t}	780±5.4	ng h mL^{-1}
$AUC_{0-\infty}$	780.3±5.4	ng h mL^{-1}
C_0	78.3±1.24	
V_d	421.5±6.6	l kg^{-1}
Cl	1.87 ±0.01	l h^{-1}

desired outcome. In parallel with chromatographic refinements, mass spectrometry optimisation was diligently executed. By directly infusing solutions of both PC and PA into the ESI source, meticulous adjustments were made to parameters such as nebulizers and desolvation gases. These refinements aimed to secure an optimal spray shape, fostering superior ionisation and droplet drying. For PC precursor ions, MH^+ is cantered at *m/z* 1326.3, with a fragment ion selected at *m/z* 574.6. In the case of PA, the precursor ion MH^+ at *m/z* 166.0 was meticulously observed, with the fragment ion registering at *m/z* 144.8. Linearity results demonstrated a direct and proportional relationship. The recovery results clearly show that the bioanalytical method works to get a high extraction rate, which supports its suitability for strong and accurate quantitative analysis. The consistently high accuracy percentages at various QC levels demonstrate the method's ability to produce reliable and consistent results. In rigorous stability

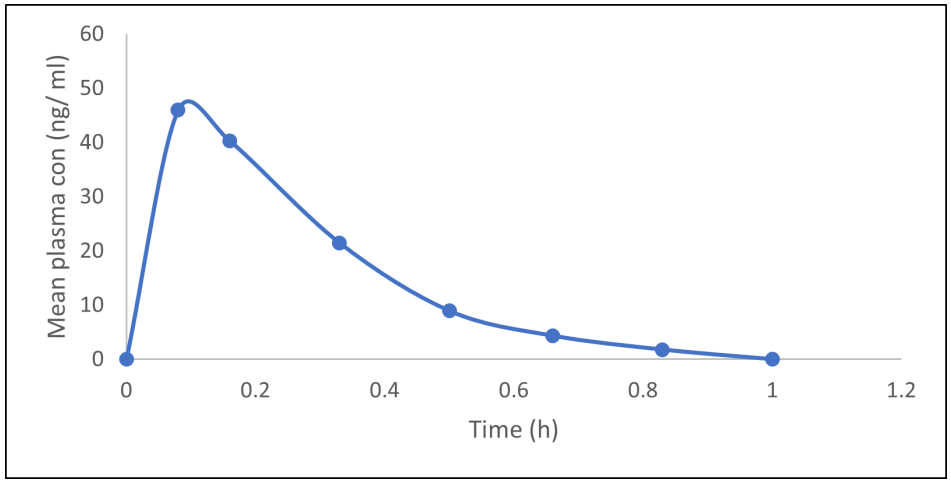

Figure 5: Graph depicting the time course of mean plasma concentrations of PC following intravenous administration in rats.

assessments, the concentrations of PC samples were observed to exhibit a variation of no more than 15% in comparison to fresh samples.

CONCLUSION

The developed method has exhibited remarkable selectivity, and linearity, along with ruggedness and reproducibility. Employing a simple liquid-liquid extraction technique with minimal matrix interference and short retention times of less than 5 m, this method ensures a relatively swift analysis process. Noteworthy are the exceptional recovery rates, reaching nearly 100% at both high and low concentrations, and the extensive stability of PC in rat plasma. The precision within and between batches %CV at (LLOQ, LQC, MQC, and HQC) levels falls below 15%. Furthermore, the current method is distinguished by its exceptional sensitivity with a LOD as low as 2 ng/mL. Notably, this bioanalytical approach, utilizing LC-MS/MS, provides the first-ever evaluation of the pharmacokinetics of PC and holds the potential to greatly facilitate the pharmacokinetic assessment of PC in rats, a crucial step in elucidating its safety, toxicity, and efficacy profiles, particularly in the context of anticancer research.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PC: Pafolacianine; **PA:** Phenylalanine; **IS:** Internal standard; **LC-MS/MS:** Liquid chromatography-mass spectrometry; **LOD:** Limits of detection; **LOQ:** Limit of quantification; **LLOQ:** Lower limit of quality control; **LQC:** Lower quality control; **MQC:** Medium quality control; **HQC:** High quality control; **ESI:** Electrospray ionization; **MRM:** Multiple Reactions Monitoring; **%RSD:** Percentage Relative Standard Deviation; **CV%:** Coefficient of variation; **ARR:** Area response ratio; $\mathbf{C_{max}}$**:** Maximum Plasma Concentration; $\mathbf{T_{max}}$**:** Time to Attain; $\mathbf{T_{1/2}}$**:** Half-life; $\mathbf{AUC_{0\text{-}t}}$**:** Area Under Curve from 0 to Time t; $\mathbf{AUC_{0\text{-}\infty}}$**:** Area Under the Curve from 0 to Infinity; **Ke:** Elimination Rate Constant; $\mathbf{C_0}$**:** Initial plasma concentration; $\mathbf{V_d}$**:** Volume of Distribution; **Cl:** Clearance Rate; ***m/z*:** Mass to charge ratio; **RPM:** Rotations per minute.

SUMMARY

The difficulties associated with early identification of ovarian cancer contribute to the disease being diagnosed at a more advanced stage. During operations to remove difficult-to-find ovarian cancer lesions, PC an optical imaging medication newly approved by the FDA under the brand name Cytalux, assists surgeons in locating and removing the affected tissue. Even if there is a lack of information regarding PC's analytical detection in biological samples, LC-MS/MS has shown that it has the potential to quantify PC in plasma. To perfect the LC-MS settings for PC analysis, one must first conduct a series of painstaking experiments using a variety of column kinds and mobile phase make-ups. The breakthrough occurred when a ratio of 40:60 acetonitrile and 0.1% formic acid was used as a buffer; this provided the best possible separation. Adjustments made to the mass spectrometry system simultaneously ensured superior ionisation and droplet drying, which led to the development of a validated analytical procedure. As a result of this method's excellent linearity, high accuracy, and stability, accurate measurement of PC concentrations in rat plasma was achieved. The pharmacokinetic profile of PC was investigated after its intravenous injection using male Sprague-Dawley rats as the test subjects. The process showcased exceptional selectivity and sensitivity, boasting a limit of detection as low as 2 ng/mL,

along with recovery rates nearing 100%. The methodology that was created is the very first pharmacokinetic study of PC, and it provides insights that are critical for comprehending both its safety and its efficacy, particularly in the context of research on anticancer treatments.

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